

Addendum

1. MONOCLONAL ANTIBODIES TO HUMAN IMMUNODEFICIENCY VIRUS AND USES THEREOF

MONOCLONAL ANTIBODIES TO HUMAN IMMUNODEFICIENCY VIRUS AND USES THEREOF

BACKGROUND OF THE INVENTION

5 Technical Field

The present invention relates to novel monoclonal antibodies which may be used in the detection of Human Immunodeficiency Virus (HIV). These antibodies exhibit an unusually high degree of sensitivity, a remarkably
10 broad range of specificity, and bind to novel shared, non-cross-reactive epitopes. In particular, the monoclonal antibodies of the present invention may be utilized to detect HIV-1 and HIV-2 core antigens in a patient sample.

15

Background Information

Acquired Immunodeficiency Syndrome (AIDS) is an infectious and incurable disease transmitted through sexual contact from HIV infected individuals or by
20 exposure to HIV contaminated blood or blood products. HIV-1 includes the formerly named viruses Human T-cell Lymphotropic Virus Type III (HTLV III), Lymphadenopathy Associated Virus (LAV), and AIDS Associated Retrovirus (ARV). HIV is a retrovirus related to a group of
25 cytopathic retroviruses, namely lentiviruses, on the basis of morphologic features, genomic organization, and nucleotide sequence (Gonda et al., Science (1985) 277:177-179; Stephan et al., Science (1986) 231:589-594; Korber, B. (ed.) et al., Human Retroviruses and AIDS. A
30 Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Published by Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, New Mexico; Reviewed in, Schochetman, G. and George, J.R., (1994) AIDS Testing. Springer-Verlag, New York,
35 Berlin, Heidelberg). HIV is an enveloped virus

00302 "SEE" 60

containing several structural proteins. Of particular relevance, the core of the virus is formed by condensation of cleavage products from a highly processed gag-pol polyprotein precursor (Pr180gag-pol) which is
 5 cleaved into a pol precursor and a gag precursor (Pr55gag). Subsequently, the core precursor Pr55gag is cleaved into p17 (myristilated gag protein), p24 (major structural protein), p7 (nucleic acid binding protein), and p9 (proline-rich protein). The envelope contains two
 10 structural proteins, gp120 (envelope glycoprotein) and gp41 (transmembrane protein) which are cleavage products of the envelope polyprotein precursor, gp160.

The most common markers of HIV infection are antibodies against viral structural proteins (Dawson,
 15 et.al., J. Infect. Dis. (1988) 157:149-155; Montagnier, et al. Virology (1985) 144:283-289; Barin, et al., Science (1985) 228:1094-1096; Schulz, T.F., et al., Lancet (1986) 2:111-112; Sarngadharan, et al., Science (1984) 224:506-508; Allan, et al., Science (1985) 228:1091-1093) and
 20 viremia in the form of detectable viral core antigen (antigenemia) (Kessler, et. al., JAMA (1987) 258:1196-1199; Phair, JAMA (1987) 258:p1218; Allain, et al., The Lancet (1986) ii:1233-1236; Kenny, et al., The Lancet (1987) 1 (8532):565-566; Wall, et al., The Lancet (1987)
 25 1(8532):p566; Stute, The Lancet (1987) 1(8532):p566; Goudsmit, et al., The Lancet (1986) ii: 177-180; vonSydow, et al., Brit. Med. J. (1988) 296:238-240; Bowen, et al. Ann. of Int. Med. (1988) 108:46-48) or detectable viral nucleic acid (Mellors, et al., Science
 30 (1996) 272: 1167-1170; Saag, et al. Nat. Med. (1996) 2: 625-629; Mulder, et al. J. Clin. Microbiol. (1994) 32:292-300; Zhang, et al., AIDS (1991) 5(6):675-681; Simmonds, et al., J. Virology (1990) 64(2):864-872). For example, in the United States, screening of blood and

009302T 000000

blood products by tests to detect antibody or antigen is mandated (Federal Food, Drug, and Cosmetic Act, 21 U.S.C. §§ 301 et. seq., Public Health Service Act 42 U.S.C. §§ 201 et. seq.). Nucleic acid testing recently has been

5 implemented in order to attain maximal reduction of the HIV seroconversion window (www.fda.gov). As a further example, various countries in Europe have begun to evaluate and use tests that detect antibody and antigen simultaneously (Ly, et al. J. Clin. Microbiol. (2000) 10 38(6): 2459-2461; Gurtler, et al., J. Virol. Methods (1998) 75: 27-38; Weber, et al., J. Clin. Microbiol (1998) 36(8): 2235-2239; Courouche', et al., La Gazette de la Transfusion (1999) N°155-Mars-Avril; Van Binsbergen, et al., J. Virol. Methods (1999) 82: 77-84), in addition to 15 European implementation of nucleic acid testing.

Serologic assays that combine antibody and antigen detection exhibit superior seroconversion sensitivity compared to assays that detect only antibody, because detection of antigen, which appears prior to antibody, 20 reduces the seroconversion window. An early version of an HIV combo assay is described in Gallarda, et al., 1992, WO93/21346, Assay for Detection of HIV Antigen and Antibody.

Within several weeks after infection with HIV, 25 individuals generally enter a clinical phase characterized by extensive viremia and acute symptoms. During this period, prior to seroconversion, HIV p24 core antigen can be detected transiently in serum or plasma specimens (antigenemia) (Devare, et al., (1990) In, Human 30 Immunodeficiency Virus: Innovative Techniques. Monograph in Virology, J.L. Melnick (ed.), Basel, Karger, vol 18: 105-121; Kessler, et al. JAMA (1987) 258: 1196-1199; Phair, J.P., JAMA (1987) 258: p1218; Allain, et al. The Lancet (1986) ii: 1233-1236; Kenny, et al., The Lancet

5.

10

25

453). However, in contrast to human (or mouse) immune polyclonal sera, mouse or human monoclonal antibodies raised or elicited against the core protein of one HIV group or type may (Mehta, et al., U.S. Patent No. 5,173,399; Butman, et al., U.S. Patent No. 5,210,181; Butman, et al., U.S. Patent No. 5,514,541) or may not (Mehta, et al., U.S. Patent No. 5,173,399; Butman, et al., U.S. Patent No. 5,210,181; Butman, et al., U.S. Patent No. 5,514,541) react against the core protein of a different HIV group or type. Often, however, neither cross-reactivity nor shared reactivity (Tijssen, 1993 In, Laboratory Techniques in Biochemistry and Molecular Biology. R.H. Burdon and P.H. van Knippenberg, eds. Vol. 15. Elsevier, Amsterdam) of mouse monoclonal antibodies have been considered or taught (Kortright, et al., U.S. Patent No. 4,888,290; Kortright, et al., U.S. Patent No. 4,886,742). In cases where HIV-1 and HIV-2 core proteins were detected simultaneously (Butman, et al., U.S. Patent No. 5,210,181; Butman, et al., U.S. Patent No. 5,514,541), a combination of at least 3 monoclonals were required, and the resulting quantitative sensitivity against HIV-1 core protein was much greater (50-fold) than for HIV-2 core protein, indicating that the monoclonals identified cross-reactive epitopes and not shared epitopes. Typically, monoclonal antibodies display a lower affinity against cross-reactive antigens (epitopes) (Karush, F. (1978) In, Comprehensive Immunology, ed. R.A. Good, S.B. Day, 5: 85-116. New York/London: Plenum; Mariuzza, et al., Rev. Biophys. Biophys. Chem. (1987) 16: 139-159; Tijssen, (1993) In, Laboratory Techniques in Biochemistry and Molecular Biology. R.H. Burdon and P.H. van Knippenberg, eds. Vol. 15. Elsevier, Amsterdam) compared to the affinity against the immunizing antigen (epitope) or shared

epitope, resulting in less sensitivity toward the cross-reactive antigen.

Shared epitopes are not readily identified, particularly within proteins of related but different sequence. A single amino acid change within an epitope can destroy or modify binding of a monoclonal antibody to that epitope (Mariuzza, et al., Rev. Biophys. Biophys. Chem. (1987) 16: 139-159). In addition, within proteins, amino acid changes (or differences) in sites outside of the epitope can change the epitope due to changes in protein folding (Mariuzza, et al., Rev. Biophys. Biophys. Chem. (1987) 16: 139-159; Laver, et al., Cell (1990) 61: 553-556), thus altering the binding of an antibody to the epitope. In this regard, the core proteins of HIV-1 group M, HIV-1 group O, and HIV-2 are related but not identical (Korber, *ibid*), and although it is known that cross-reactive epitopes exist between HIV core proteins, it is neither certain nor taught that shared epitopes are present.

The extensive genetic (and therefore antigenic) variability of HIV has not been predicted, although many scientific papers have sought to supply explanations for the mechanism(s) of variability (Meyerhans, et al., Cell (1989) 58: 901-910; Wain-Hobson, Curr. Top. Microbiol. Immunol. (1992) 176:181-193; Holland, et al., Curr. Top. Microbiol. Immunol. (1992) 176: 1-20; Gao, F. et al., Nature (1999) 397: 436-441; Sharp, et al., Biol. Bull. (1999) 196: 338-342; Robertson, et al., Nature (1995) 374: 124-126; Zhu, J. Virol. (1995) 69: 1324-1327).

Determination of HIV genetic (and therefore antigenic) variability has relied solely on many empirical observations that subsequently have led to phylogenetic classification based on variation of HIV nucleic and amino acid sequence (Korber, *ibid*). Similarly,

prediction of shared epitopes between HIV (core) proteins cannot be made because (a) core protein sequences must first be discovered, (b) once discovered, genetic variation provides added complexity and uncertainty to the identification of shared epitopes and (c) epitope discovery and characterization are required to differentiate cross-reactive from shared epitopes.

Shared epitopes between HIV-1 group M, HIV-1 group O, and HIV-2 could not be determined until the discovery of HIV-1 group O in 1994 (Gurtler, et al., J. Virol. (1994) 68: 1581-1585; Haesevelde, et al., J. Virol. (1994) 68: 1586-1596; Charneau, et al., Virology (1994) 205: 247-253).

The role of monoclonal antibody affinity for equivalent quantitative detection of variable HIV core proteins generally has not been taught (Mehta, et al., U.S. Patent No. 5,173,399; Gallarda, et al. WO93/21346; Zolla-Pazner, et al., U.S. Patent No. 5,731,189; Mestan, et al., EP 0519866A1; Butman, et al., U.S. Patent No. 5,210,181; Butman, et al., U.S. Patent No. 5,514,541; Kortright, et al., U.S. Patent No. 4,888,290; Kortright, et al., U.S. Patent No. 4,886,742). An average affinity for a monoclonal antibody elicited against a protein antigen is $4.5 \times 10^7 \text{ mol}^{-1}$ (Mariuzza, et al., Rev. Biophys. Biophys. Chem. (1987) 16: 139-159; Karush, F. (1978) In, Comprehensive Immunology, ed. R.A. Good, S.B. Day, 5: 85-116. New York/London: Plenum). Additionally, immunization strategies to increase the probability of obtaining monoclonals against shared epitopes have not been taught.

Only by combining two unpredictable features of monoclonal antibodies, affinity and shared reactivity, one can reasonably expect to obtain monoclonal antibodies which can be used to detect equivalent amounts of related but non identical HIV core proteins. Simple cross-

reactivity of monoclonal antibodies is likely to be insufficient to achieve equivalent quantitative detection of HIV core proteins. Rather, shared reactivity in combination with high affinity is required to achieve the desired result. The affinity of a monoclonal for a related core protein may be substantially lower than that determined with the immunizing core protein. In that case, the epitope is most likely cross-reactive and the affinity of the antibody for the cross-reactive epitope may severely limit the utility of the antibody for detection of diagnostically relevant (i.e., 25pg p24/ml serum or plasma, Couroucé, et al., La Gazette de la Transfusion (1999) N° 155-Mars-Avril) concentrations of the cross reactive core protein.

There are currently no known descriptions of immunoassays using only 2 monoclonal antibodies to achieve equivalent quantitative detection of HIV-1 group M, HIV-1 group O, and HIV-2 core proteins. Thus, such an immunoassay is certainly desirable. Two or more monoclonals in combination with polyclonal sera (immunoglobulin) have provided the basis for immunoassays to detect HIV-1 core protein or simultaneously HIV-1 and HIV-2 core proteins (Mehta, et al., U.S. Patent No. 5,173,399; Butman, et al., U.S. Patent No. 5,210,181; Butman, et al., U.S. Patent No. 5,514,541; Kortright, et al., U.S. Patent No. 4,888,290; Kortright, et al., U.S. Patent No. 4,886,742; Gallarda, et al. WO93/21346). Thus, in view of the above, previous literature fails to (a) describe or teach immunoassay restricted to two monoclonals for equivalent quantitative detection of HIV-1 group M and HIV-2 core proteins, (b) describe or teach immunoassays restricted to two monoclonal antibodies for equivalent quantitative detections of HIV-1 group M, HIV-1 group O, and HIV-2 core proteins, (c) teach methods to

overcome monoclonal affinity barriers recognizing cross-reactive antigens leading to non-equivalent detection of HIV-1 group M, O, and HIV core proteins, and (d) high affinity monoclonal antibodies against shared-epitopes as the methods and means to detect diagnostically relevant and equivalent amounts of non-identical core proteins from HIV-1 group M, HIV-2 group O, and HIV-2.

All U.S. patents, patent applications and publications referred to herein are hereby incorporated in their entirety by reference.

SUMMARY OF THE INVENTION

The present invention relates to monoclonal antibodies and methods of using these antibodies in the detection of Human Immunodeficiency Virus Type 1 (Groups M and O) and Type 2, the etiologic agents of Acquired Immunodeficiency Syndrome (AIDS), in serum, plasma, or other bodily fluids. In particular, the invention encompasses diagnostic methods that employ compatible, high affinity, unique mouse monoclonal antibodies identifying non-cross-reactive, shared epitopes in order to detect equivalent amounts of HIV-1 core protein (p24) and HIV-2 core protein (p26). Such antibodies also may be used in assays which detect HIV antigen and in combination assays that simultaneously detect HIV antigen and HIV antibody. In a preferred embodiment of the present invention, only two complementary, high affinity, broadly specific mouse monoclonal antibodies are required to detect equivalent amounts of core proteins from HIV-1 group M, HIV-1 group O, and HIV-2.

The monoclonal antibodies of the present invention have high affinities (K_{eq} values) sufficient to detect diagnostically relevant femtomolar quantities of HIV

core protein; however, they also possess broad specificity (i.e., shared-reactivity) for detection of equivalent quantities of related, but nonidentical, core proteins from HIV-1 group M, HIV-1 group O, and HIV-2.

In particular, the present invention encompasses monoclonal antibodies which specifically bind to Human Immunodeficiency Virus-1 groups O and M protein p24 and Human Immunodeficiency Virus-2 protein p26. These monoclonal antibodies are, for example, 120A-270, 115B-151, 103-350, 115B-303, 117-289, and 108-394. The present invention also includes the hybridomas that produce these antibodies.

Furthermore, the present invention also encompasses a method for detecting the presence of one or more antigens selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in a test sample suspected of containing one or more of the antigens. The method comprises the steps of: a) contacting the test sample with at least one monoclonal antibody (e.g., 120A-270) which specifically binds to shared epitopes on Human Immunodeficiency Virus-1 protein p24 and Human Immunodeficiency Virus-2 protein p26 for a time and under conditions sufficient for the formation of antibody/antigen complexes; and b) detecting the complexes, presence of the complexes indicating presence of at least one antigen selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in the test sample. The monoclonal of step (a) may be, for example, any one of the monoclonal antibodies described herein. It may or may not be labeled. Preferably, only one monoclonal antibody is contacted with the test sample.

The present invention also includes a method for simultaneously detecting the presence of one or more antigens selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in a test sample suspected of containing one or more of the antigens. The method comprises the steps of: a) contacting the test sample with at least one monoclonal antibody which specifically binds to Human Immunodeficiency Virus-1 protein 24 and Human Immunodeficiency Virus-2 protein p26 for a time and under conditions sufficient for the formation of antibody/antigen complexes; b) adding a conjugate to the resulting antibody/antigen complexes for a time and under conditions sufficient to allow the conjugate to bind to the bound antigen, wherein the conjugate comprises an antibody attached to a signal generating compound capable of generating a detectable signal; and c) detecting the presence of antigen which may be present in the test sample by detecting a signal generated by the signal-generating compound, presence of the signal indicating presence of at least one antigen selected from the group consisting of HIV-1 antigen and HIV-2 antigen in the test sample. The at least one monoclonal antibody of step (a) may be, for example, 120A-270, 115B-151, 117-289, 103-350, 108-394 or 115B-303. Preferably, one monoclonal antibody is used, in particular, 120A-270. The antibody of step (b) of the conjugate may be, for example, 120A-270, 115B-151, 117-289, 103-350, 108-394 or 115B-303, and is preferably 115B-151. Preferably, monoclonal antibody 120A-270 (or 117-289) and monoclonal antibody 115B-151 are used as a pair, whether 120A-270 (or 117-289) is on the solid phase or is present in the conjugate, or whether 115B-151 is on the solid phase or is present in the conjugate.

Moreover, the present invention also encompasses a method for detecting the presence of one or more antigens selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in a test sample suspected of containing one or more of these antigens, comprising the steps of: (a) simultaneously contacting: 1) at least one monoclonal antibody, which specifically binds to HIV-1 p24 antigen and HIV-2 p26 antigen, bound to a solid support, 2) the test sample, and 3) an indicator reagent comprising an antibody which specifically binds to HIV-1 antigen and HIV-2 antigen to which a signal generating compound is attached, to form a mixture; (b) incubating the mixture for a time and under conditions sufficient to form antibody/antigen/antibody complexes; (c) detecting the presence of a measurable signal generated by the signal-generating compound, presence of the signal indicating presence of one or more antigens in said test sample selected from the group consisting of HIV-1 antigen and HIV-2 antigen. The at least one monoclonal antibody of step (a) may be, for example, 120A-270, 115B-151, 117-289, 108-394, 115B-303 or 103-350, and is preferably 120A-270. The antibody of the conjugate of step (b) may be, for example, 120A-270, 115B-151, 117-289, 108-394, 115B-303 or 103-350, and is preferably 115B-151. Again, it is important to note that any one or more monoclonal antibodies of the present invention may be used on the solid phase in connection with any other monoclonal antibody of the invention (in the conjugate or solution phase). Certain pairs of monoclonal antibodies are preferred, however, and it is preferable to have only one monoclonal antibody on the solid phase.

The present invention also includes a kit for determining the presence of one or more antigens selected

from the group consisting of HIV-1 antigen and HIV-2 antigen in a test sample comprising: (a) at least one monoclonal antibody which specifically binds to Human Immunodeficiency Virus-1 protein p24 and Human Immunodeficiency Virus-2 protein p26; and (b) a conjugate comprising an antibody attached to a signal-generating compound capable of generating a detectable signal.

The at least one monoclonal antibody of (a) may be, for example, 120A-270, 115B-151, 117-289, 108-394, 115B-303, or 103-350, and is preferably 120A-270. The antibody of (b) may be, for example, 120A-270, 115B-151, 117-289, 108-394, 115B-303, or 103-350, and is preferably 115B-151.

The present invention also includes a diagnostic reagent comprising at least one monoclonal antibody selected from the group consisting of 120A-270, 115B-151, 117-289, 103-350, 108-394 and 115B-303.

Additionally, the present invention encompasses isolated epitopes or peptides having the amino acid sequences shown in SEQ ID Nos: 1-6.

The present invention also includes methods of simultaneously detecting both antigen and antibody to HIV-1 and/or HIV-2 in a patient sample. One such method involves detecting 1) one or more antibodies selected from the group consisting of HIV-1 antibody and HIV-2 antibody, and 2) one or more antigens selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in a test sample suspected of containing one or more of the antibodies and one or more of said antigens, comprising the steps of: a) contacting the test sample with at least one HIV-1 antigen which binds to HIV-1 antibody for a time and under conditions sufficient for the formation of HIV-1 antigen/HIV-1 antibody complexes; b) detecting the HIV-1 antigen/HIV-1 antibody complexes, presence of the

complexes indicating presence of HIV-1 antibody in the test sample; c) contacting the test sample with at least one HIV-2 antigen which binds to HIV-2 antibody for a time and under conditions sufficient for the formation of HIV-2 antigen/HIV-2 antibody complexes; d) detecting the HIV-2 antigen/HIV-2 antibody complexes, presence of the complexes indicating presence of HIV-2 antibody in the test sample; e) contacting the test sample with at least one monoclonal antibody which specifically binds to Human Immunodeficiency Virus-1 protein p24 and Human Immunodeficiency Virus-2 protein p26 for a time and under conditions sufficient for the formation of antibody/antigen complexes; and f) detecting the complexes, presence of the complexes indicating presence of at least one antigen selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in the test sample. Again, it is preferable to utilize certain pairs of monoclonal antibodies in connection with HIV-1 and HIV-2 antigen detection (e.g., 120A-270 and 115B-151).

Another method encompassed by the present invention involves detecting 1) one or more antibodies selected from the group consisting of HIV-1 antibody and HIV-2 antibody, and 2) one or more antigens selected from the group consisting of HIV-1 antigen and HIV-2 antigen, in a test sample suspected of containing one or more of the antibodies and one or more of the antigens, comprising the steps of: a) contacting the test sample with at least one HIV-1 antigen which specifically binds to HIV-1 antibody for a time and under conditions sufficient for the formation of HIV-1 antigen/HIV-1 antibody complexes; b) adding a conjugate to the resulting HIV-1 antigen/HIV-1 antibody complexes for a time and under conditions sufficient to allow the conjugate to bind to the bound

antibody, wherein the conjugate comprises an antigen attached to a signal- generating compound capable of generating a detectable signal; c) detecting HIV-1 antibody which may be present in the test sample by
5 detecting a signal generated by the signal-generating compound, presence of the signal indicating presence of HIV-1 antibody in the test sample; d) contacting the test sample with at least one HIV-2 antigen which specifically binds to HIV-2 antibody for a time and under conditions
10 sufficient for the formation of HIV-2 antigen/HIV-2 antibody complexes; e) adding a conjugate to the resulting HIV-2 antigen/HIV-2 antibody complexes for a time and under conditions sufficient to allow the conjugate to bind to the bound antibody, wherein the
15 conjugate comprises an antigen attached to a signal generating compound capable of generating a detectable signal; f) detecting HIV-2 antibody which may be present in the test sample by detecting a signal generated by the signal- generating compound, presence of the signal
20 indicating presence of HIV-2 antibody in the test sample; g) contacting the test sample with at least one monoclonal antibody which specifically binds to Human Immunodeficiency Virus-1 protein 24 and Human Immunodeficiency Virus-2 protein p26 for a time and under
25 conditions sufficient for the formation of antibody/antigen complexes; h) adding a conjugate to the resulting antibody/antigen complexes for a time and under conditions sufficient to allow the conjugate to bind to the bound antigen, wherein the conjugate comprises an
30 antibody attached to a signal-generating compound capable of generating a detectable signal; and i) detecting presence of antigen which may be present in said sample by detecting a signal generated by the signal-generating compound, presence of the signal indicating presence of

003436 130500

5 other pairs may also be utilized.

BRIEF DESCRIPTION OF THE DRAWINGS

10 features (e.g., helices A-J) determined from group M p24
structure are shown above the sequence alignment.

15 Figure 1b illustrates amino acid sequence alignment
of p24 from HIV-1 group M, HIV-1 group O, and HIV-2 p26.

Figure 2a and 2b illustrate the binding of monoclonal antibodies 103-350, 117-289, 115-303, 120A-270, and 115B-151 to p24 synthetic peptides.

20 Figure 3 illustrates the location of deletion clones
derived from p24 of HIV-1 group M and O.

Figure 4 illustrates the results of Western blots used to map binding of monoclonal antibodies 115B-151 and 108-394 to regions of p24.

25 Figure 5 summarizes HIV-1 p24 epitopes recognized by
p24 monoclonal antibodies.

Figure 6 illustrates HIV-1 group M p24 quantitative sensitivity achieved using 120A-270 on a solid phase and 115B-151 in solution phase.

Figure 7 illustrates HIV-1 group O p24 quantitative sensitivity achieved using 120A-270 on a solid phase and 115B-151 in solution phase.

Figure 8 illustrates HIV-2 p26 quantitative sensitivity achieved using 120A-270 on a solid phase and 115B-151 in solution phase.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel monoclonal antibodies to HIV-1 protein p24 and HIV-2 protein p26, methods for using these monoclonal antibodies, and kits containing these antibodies. More specifically, the present invention relates to monoclonal antibodies referred to herein as 120A-270 (e.g., clone 108), 115B-151 (e.g., clone 423), and 117-289 (e.g., clone 555).

Additionally, the present invention includes monoclonal antibodies referred to herein as 103-350 (e.g., clone 474), 108-394 (e.g., clone 470) and 115B-303 (e.g., clone 620).

The present invention not only includes the monoclonal antibodies referred to above but also includes the novel hybridoma cell lines which produce these antibodies. More specifically, the cell line ATCC HB ____ produces monoclonal antibody 120A-270, the cell line ATCC HB ____ produces monoclonal antibody 115B-151, the cell line ATCC HB ____ produces monoclonal antibody 117-289, the cell line ATCC HB ____ produces monoclonal antibody 103-350, the cell line ATCC HB ____ produces monoclonal antibody 108-394, and the cell line ATCC HB ____ produces monoclonal antibody 115B-303. The cell lines producing the antibodies were deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110 under the terms of the Budapest Treaty on _____, 2000 and were accorded the ATCC numbers noted above.

The monoclonal antibodies of the present invention or fragments thereof may be used in immunoassays for the

009027 SEP 1980

detection of HIV-1 (Groups M and O) and HIV-2, simultaneously. (For purposes of the present invention, a "fragment" is defined as a subunit of the monoclonal antibody which reacts in the same manner, functionally, as the full antibody with respect to binding properties.) In particular, when monoclonal antibodies 120A-270 and 115B-151, or monoclonal antibodies 117-289 and 115B-151 are used in combination in an immunoassay, for example, in a sandwich assay, one may minimally detect core antigen (p24) from subtypes A, B, C, D, E, F, G and O of HIV-1 groups M and O, and HIV-2 core antigen (p26) in a patient sample. In fact, less than 25 picogram (i.e., picogram core antigen/ml of serum or plasma) quantities of the HIV-1 p24 antigen and HIV-2 p26 antigen may be detected using the combinations of monoclonal antibodies described above. Thus, the monoclonal antibodies of the present invention have a high degree of sensitivity as well as broad specificity. In particular, the unique property of these antibodies is that they recognize related, but non-identical, core antigens with approximately equivalent affinity (i.e., equivalent quantitative sensitivity), indicating that they recognize unpredictable shared epitopes, and thus exhibit shared reactivity, rather than typical and expected cross-reactive epitopes and thus exhibiting cross-reactivity. (For purposes of the present invention, "cross-reactivity" is defined as the binding of an antibody to structurally different determinants on different antigens. Antibody affinity for a cross-reactive epitope (i.e., antigen) is lower than that for the immunogenic epitope (i.e., antigen) or shared epitope. "Shared reactivity" is defined as the binding of an antibody to structurally identical determinants on different antigens. Antibody affinity for a shared epitope is

equivalent to the affinity for the immunogenic epitope (i.e., immunogen).) It should also be noted that the pairs of monoclonal antibodies are compatible, that is, each monoclonal antibody of the pair maps to a different epitope or antigenic determinant on the core protein(s). Binding of one antibody of the pair does not interfere with binding of the second antibody of the pair.

In one embodiment of the invention, the preferred embodiment, monoclonal antibody 120A-270 or a fragment thereof is coated onto a solid phase (e.g., a microparticle, a microtiter well, a bead, etc.); however, 115B-151 or 117-289 may also be used or fragments thereof. The test sample is then contacted with the monoclonal antibody or fragment thereof such that, if p24 antigen or p26 antigen is present in the patient sample, antibody/antigen complexes are then formed as a first mixture. (For example, both monoclonal antibody/p24 antigen and monoclonal antibody/p26 antigen complexes may be formed if the patient has both HIV-1 and HIV-2.) One then adds a conjugate comprising (a) a probe antibody, for example, monoclonal antibody 115B-151 (which binds an epitope distinct from and compatible with the epitope bound by 120-270) attached to (b) a signal-generating compound. Antibody/antigen/antibody probe complexes are then formed as a second mixture. HIV-1 and/or HIV-2 antigen is then detected in the sample by detecting the presence of the signal generated and thus the antibody/antigen/antibody probe complexes. The amount of antigen(s) in the test sample may also be calculated, as the signal generated is proportional to the amount of antigen in the sample.

Another manner of detecting the complexes formed is to utilize a conjugate comprising a third antibody attached to a signal-generating compound. In particular,

once the antibody/antigen/antibody complexes described above have formed (i.e., the latter antibody being the 2nd antibody which is unlabelled), one may then add a conjugate which binds to the "2nd" unlabelled antibody in solution. The conjugate may comprise, for example, an antigen or anti-antibody capable of binding to the bound second antibody (e.g., anti-115B-151 antibody or an antibody to the probe antibody) attached to a signal-generating compound capable of generating a detectable signal. Detection of the signal thus indicates presence of the complexes and thus presence of the antigen in the sample. The signal generated is actually proportional to the amount of antigen present in the sample. (See, e.g., U.S. Patent No. 6,015,662.) The design of the assay is dependent upon the affinities and specificities of the antibodies used, accuracy of results obtained, convenience, the nature of the solid phase, etc. (See U.S. Patent No. 5,104,790 for a discussion of different antigen assay formats.)

Additionally, it should also be noted that the initial capture antibody used in the immunoassay may be covalently or non-covalently (e.g., ionic, hydrophobic, etc.) attached to the solid phase. Linking agents for covalent attachment are known in the art and may be part of the solid phase or derivatized to it prior to coating. Examples of solid phases used in immunoassays are porous and non-porous materials, latex particles, magnetic particles, microparticles, beads, membranes, microtiter wells and plastic tubes. The choice of solid phase material and method of labeling the antigen or antibody present in the conjugate, if desired, is determined based upon desired assay format performance characteristics.

As noted above, the conjugate (or indicator reagent) will comprise an antibody (or perhaps anti-antibody,

depending upon the assay), attached to a signal-generating compound or label. This signal-generating compound or "label" is in itself detectable or may be reacted with one or more additional compounds to generate a detectable product. Examples of signal-generating compounds include chromogens, radioisotopes (e.g., ^{125}I , ^{131}I , ^{32}P , ^3H , ^{35}S and ^{14}C), chemiluminescent compounds (e.g., acridinium), particles (visible or fluorescent), nucleic acids, complexing agents, or catalysts such as enzymes (e.g., alkaline phosphatase, acid phosphatase, horseradish peroxidase, beta-galactosidase and ribonuclease). In the case of enzyme use (e.g., alkaline phosphatase or horseradish peroxidase), addition of a chromo-, fluoro-, or lumino-genic substrate results in generation of a detectable signal. Other detection systems such as time-resolved fluorescence, internal-reflection fluorescence, amplification (e.g., polymerase chain reaction) and Raman spectroscopy are also useful.

Another type of assay in which the present monoclonal antibodies may be utilized involves simultaneously contacting: 1) one monoclonal antibody (bound to a solid support), 2) the test sample and 3) an indicator reagent comprising a monoclonal antibody or fragment thereof (e.g., 115B-151, which specifically binds to HIV-1 and HIV-2 antigen) to which a signal generating compound is attached, to form a mixture. The mixture is then incubated for a time and under conditions sufficient to form antibody/antigen/antibody complexes. The presence, if any, of HIV-1 and/or HIV-2 antigen present in the test sample and captured on the solid phase is determined by detecting the measurable signal generated by the signal-generating compound. The amount of antigen present in the test sample is proportional to the signal generated. In this assay or those described

above, the monoclonal antibodies of the present invention may be used either as the capture phase or as part of the indicator reagent in solution (i.e., the reagent comprising an antibody and a signal-generating compound).

5 Such diagnostic procedures, including those described above and below, are well-known in the art (see Immunological Methods, Vols. I and II, 1979 and 1981, Eds., Lefkovits and Pernis, Academic Press, New York; Monoclonal Antibodies, 1982, eds., Kennett et al., Plenum Press, New York; and Handbook of Experimental Immunology, 10 1978, ed., Weir, Blackwell Scientific Publications, St. Louis, MO).

It should be noted that the monoclonal antibodies of the present invention preferably may be used either
15 alone, as a single capture antibody, or alone as a single probe and/or conjugated antibody. However, they may also be used in pairs or in trios in the assays described above. Further, combinations of the monoclonal antibodies of the present invention (and fragments
20 thereof) may be used with other monoclonal antibodies that have specificities for epitopes of HIV-1 and/or HIV-2, other than the epitope specificities of the monoclonal antibodies of the present invention. Thus, the present monoclonal antibodies may act as components in a mixture
25 or "cocktail" of HIV-1 and/or HIV-2 antibodies. Thus, for example, this cocktail can include a monoclonal antibody of the present invention which detects p24 of HIV-1 and p26 of HIV-2 (e.g., 120A-270) and a monoclonal antibody which detects a HIV envelope antigenic
30 determinant in the transmembrane protein or extracellular glycoprotein. In this manner, one may be able to detect several antigenic determinants from different proteins of one or more viruses (e.g., HIV-1 and HIV-2) simultaneously.

Also, it should be noted that the monoclonal antibodies of the present invention may be utilized in a combination assay which detects: 1) antigens, such as those described above (e.g., p24 and p26) and 2) antibodies to HIV (by use of, for example, envelope antigens (e.g., HIV-1 group M and O gp41 and HIV-2 gp36). Any such combination assay, which utilizes the monoclonal antibodies of the present invention, is considered to be within the scope of the invention.

Examples of biological fluids which may be tested by the above immunoassays include plasma, serum, cerebrospinal fluid, saliva, tears, nasal washes or aqueous extracts of tissues and cells. The test samples may also comprise inactivated whole virus or partially purified or recombinant p24 or p26 antigen.

It should also be noted that the above-referenced monoclonal antibodies may be used, when appropriately labeled, as competitive probes against HIV-1 and -2 core antibodies in serum samples for binding to recombinantly-derived HIV-1 p24 and HIV-2 p26.

Additionally, the monoclonal antibodies of the present invention or fragments thereof may be used in detection systems using fixed cells or fixed tissues, with appropriate labeling of each monoclonal antibody. In particular, the tissue sample is contacted with a conjugate comprising a signal-generating compound attached to one of the monoclonal antibodies of the present invention in order to form a mixture. The mixture is then incubated for a time and under conditions sufficient for antigen/antibody complexes to form. The presence of antigen present in the sample is determined by detecting the signal generated. The antibodies may also be utilized for purifying HIV-1 p24 antigen and HIV-2 p26 antigen by, for example, affinity chromatography.

Furthermore, the antibodies of the invention may be bound to matrices and used for the affinity purification of specific HIV-1 and/or HIV-2 antigens from, for example, cell cultures, or biological tissues such as blood and liver. The monoclonal antibodies, for example, may be attached to or immobilized on a substrate or support. The solution containing the HIV antigenic determinants is then contacted with the immobilized antibody for a time and under conditions suitable for the formation of immune complexes between the antibody and polypeptides containing the p24 and p26 determinants. Unbound material is separated from the bound immune complexes. The complexes or antigenic fragments are then separated from the support.

One or more of the monoclonal antibodies of the present invention, and preferably the pairs suggested above, is particularly suitable for use in the form of a kit. The kit may comprise one or more containers such as vials or bottles, with each container containing a pair of the monoclonal antibodies, or as cocktails of monoclonal antibodies. These kits may also contain vials or containers of other reagents needed for performing the assay, such as washing, processing and indicator reagents.

Additionally, the present invention also includes a vaccine comprising one or more of the monoclonal antibodies of the present invention and a pharmaceutically acceptable adjuvant (e.g., Freund's adjuvant) which can be administered to HIV-infected individuals (i.e., passive immunization). Furthermore, the monoclonal antibodies of the present invention can serve prophylactically for administration to non-infected, high-risk individuals, such as health care workers.

It should also be noted that the monoclonal antibodies of the present invention may also serve as research tools for epitope mapping of HIV proteins p24 and p26. Further, it should be noted that not only do the monoclonal antibodies of the present invention bind to proteins and protein precursors of HIV clinical isolates which contain the targeted region or regions of antigenic determinants, in addition, the antibodies bind to recombinant proteins and synthetic analogues of the proteins which contain the antigenic determinant(s). Thus, for example, the monoclonal antibodies of the present invention may be used in binding experiments involving recombinant proteins and synthetic analogues of p24 of HIV-1 and p26 of HIV-2.

Additionally, antibodies of the present invention which are unlabeled may be used in agglutination assays or can be used in combination with labeled antibodies that are reactive with the monoclonal antibody, such as antibodies specific for immunoglobulin.

The present invention also comprises a method for treating a mammal infected with HIV-1 and/or HIV-2 comprising administering to a mammal, in need of such treatment, an effective amount of one or more of the monoclonal antibodies of the present invention in the form of a pharmaceutical composition, as described directly below. A pharmaceutically effective amount means any amount of the compound which, when incorporated in the pharmaceutical composition, will be effective to inhibit HIV replication and thereby treat Acquired Immunodeficiency Syndrome (AIDS) but less than an amount which would be toxic to the subject.

Additionally, the present invention encompasses pharmaceutical compositions comprising one or more of the monoclonal antibodies of the present invention and a

pharmaceutically acceptable carrier. A pharmaceutical carrier is any compatible, non-toxic substance suitable to deliver one or more monoclonal antibodies to the patient. For example, sterile water, alcohol, fats, waxes and inert solids may be used as carriers. The composition may also contain monoclonal antibodies which bind to proteins or glycoproteins of HIV other than p24 and/or p26. Further, the pharmaceutical composition may be administered alone or in conjunction with other anti-retroviral agents. (See Mitsuya et al., Nature 325:773-778 (1987).) The pharmaceutical compositions of the present invention may be administered either orally or parenterally (i.e., subcutaneously, intramuscularly or intravenously).

Further, it should be noted that one or more of the monoclonal antibodies of the present invention may be used to generate chimeric antibodies for therapeutic use, for example, or as assay controls or calibrators.

Since all of the monoclonal antibodies of the present invention bind both to p24 of HIV-1 and to p26 of HIV-2, as evidenced by the data presented in Table 5, for example, any one or more of the monoclonal antibodies may be used in the diagnostic assays, kits, compositions and methods described above. Certainly those with the strongest binding specificities and capabilities (with respect to p24 and p26) are preferred.

The present invention may be illustrated by the use of the following non-limiting examples:

Example 1

Immunogen Selection

The immunization strategies included HIV-1 group O and HIV-1 group M antigens to drive the immune response toward recognition of both shared epitopes within the

core antigens of both groups of HIV-1. Three different HIV-1 immunogens were used in various combinations to develop an anti-HIV-1 p24 response in the animal host. Two HIV-1 group M antigens manufactured at Abbott Laboratories (Abbott Park, IL) were derived from denatured whole viral lysates while native HIV-1 group M p24 (p24M) protein was purified from the viral lysates.

The third immunogen was a recombinant p24 antigen (rp24-O) derived from the gag gene of HIV-1 group O isolate HAM112. The p24 gene from HAM112 was cloned into the lambda PL vector and expressed in *E. coli*. The construction, scale up and purification of the recombinant antigen were performed according to published methods for recombinant proteins made in *E. coli*. (Seetharam, R. and Sharma, S.K., (eds), 1991. 'Purification and Analysis of Recombinant Proteins', Marcel Dekker. New York, NY.) Verification of the amino acid sequence against published results confirmed integrity of the product. (Van den Haesevelde et al., 1994. *Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate*. J. Virol. 68:1586.)

Example 2

Immunization of Mice

The animal models selected for hybridoma development were three strains of mice, the CAfl, the RBf/dn, and the BALB/c. The mice were females, age 6-8 weeks old, purchased from Jackson Laboratory (Bar Harbor, Maine). In order to produce anti-HIV-1 p24 monoclonal antibodies with high affinity, two different immunization strategies were utilized. Hybridomas secreting anti-p24 monoclonal antibodies (Mab) 103-350-474, 108-394-470, 115B-303-620, 115B-151-423, and 117-289-555 were produced from mice

[illegible]

The immunization procedures are described in detail as follows:

Hybridoma 108-394-470 was produced from cell fusion
#108. On day 1, CAF1 mouse #1556 received 10 ug of rp240
antigen in 0.2 ml of CFA given s.c. On day 56, the mouse
received 10 ug of rp240 antigen in 0.2 ml of IFA given
i.m. On day 74, the mouse was bled for assessment of
anti-HIV-1 antibody titer by indirect EIA. On day 270,
the mouse was boosted i.s. with 45 ug of purified native
HIV-1 group M p24 in normal saline.

Hybridomas 115B-151-423 and 115B-303-620 were produced from cell fusion #115B. On day 1, CAF1 mouse #1563 received 10 ug of rp240 antigen in 0.2 ml of CFA

given s.c. On day 210, the mouse received mixture of 10 ug of rp240 and 10 ug of p24M in 0.2 ml of IFA given s.c. On day 235, the mouse was bled for assessment of anti-HIV-1 antibody titer by indirect EIA. On day 375, the mouse was boosted i.s. with a mixture of 10 ug of rp240 and 10 ug of p24M in normal saline.

Hybridoma 117-289-555 was produced from cell fusion #117. On day 1, Rbf/dn mouse #1545 received 10 ug of rp240 antigen in 0.2 ml of CFA given s.c. On day 56, the mouse received mixture of 10 ug of rp240 in 0.2 ml of IFA given i.m. On day 74, the mouse was bled for assessment of anti-HIV-1 antibody titer by indirect EIA. On day 392, the mouse was boosted i.s. with a mixture of 45 ug of rp240 and purified native p24M in normal saline.

Hybridoma 120A-270-108 was produced from cell fusion #120A of a hyperimmunized BALB/c mouse #7 which received 0.2 ml of immunogen containing 10 ug of purified native p24M antigen and 4 ug of S. typhimurium extract (RIBI Immunochemicals, RiBi Immuno Chem Research, Hamilton, Montana) given i.p. on day 1, s.c. on day 7, and i.p. on day 14. On day 21, the mouse was bled for assessment of anti-HIV-1 p24 antibody titer by indirect EIA. On days 28, 35, and 42, the mouse received 5 ug of purified native p24M antigen in 0.2 ml of RIBI adjuvant given s.c., i.p., and s.c., respectively. On day 49, the mouse was bled a second time for assessment of anti-HIV-1 antibody titer by indirect EIA. On day 77, three days before fusion, the mouse was boosted i.s. with 50 ug of purified native p24M antigen in normal saline.

Example 3

Assessment of Anti-p24 Antibody Titer of the Immunized Mice

Indirect binding and direct sandwich enzyme-linked immunoassays (EIA) were used to assess anti-HIV-1

antibody titers from the immunized mice. (Direct sandwich EIA was performed with a limited amount of core antigen to detect only high affinity antibodies.) Sera from naïve or immunized mice were serially diluted in 10 mM sodium phosphate buffer (PBS), pH 7.4, containing 5% bovine serum albumin (BSA) and 0.03% sodium azide as preservative. The detailed assay procedures are described below. The assessment of anti-p24 antibody titers from the immunized mice is shown in Table 1a from the indirect binding EIA and Table 1b from the direct sandwich EIA.

Table 1a. Assessment of anti-p24 antibody titers by indirect EIA

A490nm reading

Fusion #	Animal ID	Sera dilution	rp24-O	p24-M	BSA control
103	CAf1 #1555	1/24,300	1.219	1.156	0.008
108	CAf1 #1556	1/24,300	1.157	1.109	0.005
117	RBf/dn #1545	1/900	0.462	0.600	0.019
120A	BALB/c #7	1/100,000	NT	1.709	0.025
	Pre-bled ms serum	1/900	0.036	0.093	0.018

Table 1b. Assessment of anti-p24 antibody titers by direct sandwich EIA

A490nm reading

Fusion #	Animal ID	Sera dilution	M lysate	O lysate
115B	CAf1 #1563	1/8,100	0.540	0.401
120A	BALB/c #7	1/1,000	1.579	0.762
	Pre-bled ms serum	1/1,000	0.163	0.162

- 5 For the direct binding EIA, briefly, the diluted sera were reacted with microtiter wells directly coated with 100 ul of 3 ug/ml in PBS of p24M (i.e., p24 from group M) or rp240 (i.e., recombinant p24 from group antigen or mixture of p24M and rp240 and then blocked
- 10 with 2% bovine serum albumin (BSA) in PBS. After 1 hour incubation at room temperature (RT) on a microtiter plate shaker (Lab-Line Instruments, Melrose, IL), the plate was washed 3 times with distilled water using a microtiter plate washer (Skanwash, Skatron Instruments, Sterling,
- 15 VA). One hundred ul of 0.2 ug/ml of goat anti-mouse IgG+IgM-Horseradish Peroxidase (HRPO) (KPL, Gaithersburg, MD) conjugate were added to each well of the plate. After incubating 30 minutes at RT, the plate was washed 3 times (as above). Enzyme substrate o-
- 20 phenylenediamine:2HCl (OPD) solution was added to each well to develop a color reaction for 5 minutes in the dark at RT. The reaction was stopped by addition of 1N H₂SO₄ into each well. The plate was read at A490nm in a microtiter plate reader (Titertek multiwell EIA reader,
- 25 ICN, Huntsville, AL).

For the indirect sandwich EIA, a microtiter wells were coated with 100 ul per well of 10 ug/ml in PBS of

goat anti-mouse IgG+M antibodies (KPL) overnight at 2-8C. The plate was washed 3 times with distilled water using a plate washer (Skanwash, Skatron Instruments, Sterling, VA) and then blocked with 2% BSA in PBS for 30 minutes at RT. One hundred ul portions of culture fluids were added to the wells, and the plate was incubated for 1 hour at RT on a plate shaker. Anti-p24 antibodies secreted in culture fluids were captured by goat anti-mouse IgG+M coated on solid phase. After washing, 100 ul portions of 100 pg/ml of HIV-1 viral lysate were added into each well and the plate was incubated for one hour at RT on a plate shaker. After washing, 100 ul portions of 0.5 ug/ml of rabbit anti-p24 antibodies were added into each well and the plate was incubated for one hour at RT on a plate shaker. After washing, 100 ul of 0.2 ug/ml of goat anti-rabbit IgG-HRPO (KPL) were added to each well, and the plate was incubated for 30 minutes at RT. After the final washing, chromogen OPD was added as described above.

Example 4

Cell Fusion

Three days after the pre-fusion antigen boost, mice were sacrificed and their spleens were disrupted to single cells. The single cell suspensions were treated with 0.83% NH_4Cl to remove red blood cells, and then mixed with SP2/0 cells at a 10:1 ratio of SP2/0:spleen cells. The mixed cells were centrifuged, washed once with serum-free medium, then centrifuged again. The supernatant was removed from the cell pellet. The fusogen, polyethylene glycol (PEG), was used to form hybrids of immune spleen cells with myeloma cell line SP2/0 (HPRT neg.) [Kohler and Milstein, Nature (1975) 256:494, and reviewed in *Monoclonal Hybridoma Antibodies: Techniques and Applications* ed. Hurrell (CRC Press, Inc., 19820].

003416 120600
003021 021200

Briefly, fusion of spleen cells and SP2/0 cells was accomplished by exposing the pellet to 40% PEG (M.W. 1450, American Type Culture Collection, Manassas, VA) in serum-free Iscoe's Modified Dulbecco's Medium (IMDM) for two minutes. The PEG and cell suspension was diluted slowly by the addition of 20 ml of serum free IMDM over a period of five minutes, followed by collection of the cells by centrifugation. The supernatant was decanted and replaced with 30 ml IMDM containing 20% fetal bovine serum (Hyclone, Logan, Utah) with HAT (hypoanthine, aminopterin, and thymidine) to select for hybridomas. Spleen cells from one nonimmunized BALB/c mouse also were added as a feeder layer. The cells were plated at 0.1 ml/well in three 96 well tissue culture plates. Three days later an additional 0.1 ml of HAT media was added to each well. At weekly intervals thereafter, one half of the media was replaced with IMDM containing 20% fetal bovine serum with HAT, and hybrids were allowed to grow an additional 7-14 days.

Some of the hybrids were composed of spleen cells, making antibody to HIV-1, fused with SP2/0 cells. Briefly, the fusogen promotes fusion of spleen cell and SP2/0 cell membranes, forming a heterokaryon containing nuclei of both cells. Eventually, the dissimilar nuclei fuse producing a single nucleus capable of synchronous mitosis. As the fused cells divide, the hybrid stabilizes by losing chromosomes of each nucleus. The fused cells were plated into multiple 96 well plates at 10^5 to 10^6 cells per well. Hybrid cells formed from SP2/0:spleen cell fusions were selectively propagated by culturing in HAT medium. All unfused SP2/0 or SP2/0:SP2/0 fused cells were prevented from growing by aminopterin, and unfused spleen cells or spleen:spleen

fused cells died off in culture. Only spleen cell:SP2/0 hybrids will grow in the HAT selection medium.

Example 5

5 Screening, Cloning and Characterization of p24 Monoclonal Antibodies

After 10-14 days, culture fluids from wells containing hybridoma cell growth were screened for
10 antibody to HIV-1 p24. The indirect EIA was used to screen the culture fluids from cell fusions #103, #108, #115B, and #117. In order to select anti-HIV core protein monoclonal antibodies with high affinity, a direct sandwich EIA assay was also utilized to screen
15 potentially useful clones from cell fusions #120A and cloning of fusions #115B and 120A. Both direct and indirect EIAs are described in the section of antisera titer assessment of Example 2. The primary screening data from the hybridomas described in this application
20 are shown in Table 2a and 2b.

003027 32460

Table 2a. Primary Fusion screened by indirect binding EIA

A490nm reading

Hybrid #	HIV Ag used	Sample	Blank control
103-350	p24-M lysate	0.921	0.030
108-394	p24-M lysate	0.497	0.000
115B-151	p24-M lysate	0.662	0.012
115B-303	p24-M lysate	0.467	0.003
117-289	p24-M lysate	0.295	0.000

5 Table 2b. Primary Fusion screened by direct sandwich EIA

A490nm reading

Hybrid #	HIV Ag used	Sample	Negative control
120A-270	p24-M lysate	0.501	-0.011

10 Hybridomas showing strong positive signal in the primary screening EIAs were transferred into 24-well plates for cell expansion. Culture fluids were again assayed for the presence of anti-p24 antibody. Anti-p24 positive hybrids were further expanded in T25 flask for cloning by limiting dilution. Each expanded hybrid was
 15 plated in a 96-well plate at a dilution of 10^5 to 10^6 and allowed to grow 10-21 days. Culture fluids from limiting dilution were assayed for the presence of anti-p24 antibody. The hybridoma designation is based on a numbering system using 3 numbers: the first being the
 20 fusion number, the second is the parental hybrid number and the third is the subclone number. Each 96-well

tissue culture plate is sequentially numbered 1 to 96. For example, hybridoma # 103-350-474 originates from the 103rd fusion. The parental hybrid is #350 as it derives from the 3rd fusion plate in well #50. The subclone is #474 since it is from the 4th cloning plate, well #74. The clones were obtained by limiting dilution using the guidelines outlined by J. W. Goding in *Monoclonal Antibodies: Principles and Practice* (Academic Press, N.Y., 1983). The primary cloning data for the hybridomas described in this application are shown in Table 3a and 3b.

Table 3a. Primary clone screened by indirect binding EIA

A490nm reading

Clone #	HIV Ag used	Sample	Blank control
103-350-474	p24-M lysate	0.489	0.021
108-394-470	p24-M lysate	0.466	0.000

Table 3b. Primary clone screened by direct sandwich EIA

A490nm reading

Clone #	HIV Ag used	Sample	Blank control
115B-151-423	p24-M lysate	0.846	0.000
115B-303-620	p24-M lysate	0.991	0.006
117-289-555	p24-M lysate	0.830	0.011
120A-270-108	p24-M lysate	0.371	-0.021

Isotypes of anti-p24 Mabs were determined with the SBA Clonotyping System (Southern Biotechnology

Associates, Inc., Birmingham, AL). Briefly, microtiter wells were coated with 100 ul portions of goat anti-mouse IgG+M antibodies (KPL) for 18-24 hours at 2-8C. The wells were washed and blocked with 2% BSA in PBS for 30 minutes at RT. After washing, 100 ul portions of culture fluids were added into the wells and incubated for 2 hour at RT on a plate shaker. After washing, 100 ul portions of rabbit anti-mouse isotype-specific antibodies were added to the wells and incubated for one hour at RT on a plate shaker. After washing, 100 ul of goat anti-rabbit IgG-HRPO (KPL) were added to the wells and incubated for another 30 minutes at RT on a plate shaker. After the final washing, chromagen OPD was added as described above. The isotypes of Mabs 103-350-474, 108-394-470, 115B-151-423, 115B-303-620, 117-289-555, and 120A-270-108 are summarized in Table 4.

Table 4. Monoclonal antibody isotypes

Mab ID	Isotype	Light chain
103-350-474	IgG2a	kappa
108-394-470	IgG2b	kappa
115B-151-423	IgG1	kappa
115B-303-620	IgG2b	kappa
117-289-555	IgG1	kappa
120A-270-108	IgG1	kappa

The specific reactivities of Mabs to HIV-1/2 antigens were tested in the direct sandwich EIA as described in the previous section. The results are summarized in Table 5.

Table 5. Reactivities of anti-HIV-1 p24 Mabs with HIV-1/2 p24/p26

Mab ID	Reactivity with		
	p24-M	p24-O lysate	HIV-2 lysate
103-350-474	++	++	++
108-394-470	++	++	+
115B-151-423	++	++	++
115B-303-620	++	++	++
117-289-555	++	++	++
120A-270-108	++	++	++

++ = Very Strong

In view of the results presented in Table 5, all of the monoclonal antibodies of the present invention react with HIV-1/2 p24/p26 antigens. (HIV-1 group O and HIV-2 NIH-Z viral lysates were purchased from ABI (Gaithersburg, MD).)

Example 6

Antibody Production and Purification

In order to produce large amounts of Mabs for further characterization and testing, anti-p24 hybridoma cell lines were further expanded in T250 flasks and weaned to serum free media, H-SFM (Life Technologies, Grand Island, NY). When the hybridoma cell lines were adapted to H-SFM, they were seeded in roller bottles for large scale antibody production. Culture fluids were harvested from the roller bottles and concentrated by a filtration system. The roller bottle derived antibody

was purified on a Protein A column from PerSeptive Biosystems (Cambridge, MA).

Example 7

5 Affinity Measurement of Anti-p24 Monoclonal Antibodies

 Affinities of purified anti-p24 Mabs 103-350-474,
108-294-470, 115B-303-620, 115B-151-423, 117-289-555, and
120A-270-108 were measured by a surface plasma resonance
(SPR) based BIAcore immunosensor instrument (Pharmacia,
10 Uppsala, Sweden). Briefly, goat anti-mouse IgG (Fc)
antibodies were covalently coupled to amino-sensor chips
by EDAC chemistry. Each mouse IgG monoclonal antibody
was injected into the sensor chip and captured by the
immobilized goat anti-mouse IgG antibodies. The unbound
15 mouse monoclonal antibody was washed away from the chip.
A baseline measurement of surface plasma resonance (SPR)
signal was recorded for each monoclonal. When purified
HIV-1 p24 protein was injected into the sensor chip and
reacted with anti-p24 monoclonal antibody, SPR signal
20 started to increase. The slope of binding curve was
proportional to the association constant of each
monoclonal antibody. After binding was achieved, a wash
step was introduced. The dissociation rate of p24 from
anti-p24 monoclonal antibody was proportional to the
25 decrease of SPR signal. After each cycle of measurement,
HCl buffer was applied into the sensor chip to remove
anti-p24 monoclonal antibody from the sensor chip for the
next measurement. Based upon the on-rate and off-rate
SPR signals, association (K_a), dissociation (K_d), and
30 relative affinity (K) constants of each monoclonal
antibody were determined. The data are summarized in
Table 6.

Table 6. Binding constants

Clone #	Ka(M-1s-1)	Kd(s-1)	K(M-1)
103-350-474	8.3 x 10e5	5.1 x 10e-4	1.6 x 10e9
108-394-470	8.1 x 10e5	4.2 x 10e-4	1.9 x 10e9
115B-151-423	1.3 x 10e6	2.0 x 10e-4	6.5 x 10e9
115B-303-620	8.3 x 10e5	2.6 x 10e-4	3.2 x 10e9
117-289-555	3.5 x 10e5	3.3 x 10e-4	1.1 x 10e9
120A-270-108	8.1 x 10e5	8.8 x 10e-4	9.2 x 10e9

Example 8Epitope Mapping of p24 Monoclonal Antibodies

Epitopes on HIV-1 p24 recognized by mouse monoclonal antibodies were identified using two sets of thirteen p24 synthetic peptides (Figure 1a). Peptide design was based on the three dimensional structure of p24 antigen (Gitti, et al., Science 273: 231 (1996); Gamble, et al., Science 278: 849 (1997)) in order to present selected, uninterrupted regions of helical structure (in vivo) that might be unique to shared epitopes. These peptides covered all helical regions (A-J) on the core proteins. Monoclonal antibodies were reacted against both group M (clade B) and group O (Ham112) peptides. Peptides were designated M1 to M13 representing HIV-1 group M clade B p24, or O1 to O13 representing HIV-1 group O (Ham 112 isolate) p24. Each peptide also contained an additional cysteine on its C-terminus which was reacted with maleimide-modified Keyhole Lympet Hemocyanin (KLH) to

form two series of KLH conjugated peptides in addition to unconjugated peptides. KLH conjugated peptides were generated in order to help stabilize and present conformational structures that might be essential for epitope presentation and recognition (monoclonal binding). KLH conjugated peptides were designated as KM1 to KM13 for group M peptides or KO1 to KO13 for group O peptides.

Binding of monoclonal antibodies (Mabs) to sets of synthetic peptides was determined by an indirect EIA assay. Briefly, free or KLH conjugated synthetic peptides were coated on the wells of microtiter plates. The peptide coated wells were incubated with Mabs prepared to a concentration of approximately 1 ug/ml. Bound Mabs were detected by enzyme or acridinium-labeled goat anti-mouse IgG antibodies. Representative data are depicted in Figures 2a and b. Epitopes recognized by Mab 103-350-474, 117-289-555, 115B-303-620 and 120A-270-108 were identified as follows:

Monoclonal antibody 103-350-474 specifically bound to the KLH conjugated group M and group O peptides # 4 (KM4/KO4), which corresponds to the helix D region of p24. The epitope is linear with apparent secondary conformational requirements. Little or no consistent binding was detected when 103-350-474 was reacted against free (un-conjugated) peptides. In contrast, when peptides were conjugated to a carrier protein (KLH) in order to promote secondary conformational peptide structures, 103-350-474 consistently exhibited strong (high S/N) binding against KM4/KO4 peptides. The epitope appears to be linear because the antibody binds small synthetic peptides, but the optimal epitope may require specific secondary helical structures. Therefore, the

epitope is most broadly defined as comprising amino acids 63-89, and most narrowly estimated as requiring amino acids 63-80, which map to the helix-D region. Further, cross reactivity of the antibody between M and O p24 indicates that the p24 regions of greatest sequence homology in helix-D are most likely involved in forming the epitope. The residues in bold are most likely key to forming the epitope, but secondary structure involving or requiring neighboring amino acids cannot be excluded.

(K)M4 peptide 63-89:

CQAAMQ **MLKET** **INEEA** **AEWDR** **VHPVH** AG (SEQ ID NO:1)

(K)O4 peptide 63-89:

CQGALQ **VLKEV** **INEEA** **ADWDR** **SHPPV** VG (SEQ ID NO:2)

Monoclonal antibody 117-289-555 specifically bound to both group M and group O M10/O10 peptides, which correspond to the helix H region, which is part of the major homology region (MHR). The epitope appears to be linear because the antibody readily binds free (un-conjugated) M10/O10 peptides. Therefore, the epitope of Mab 117-289-555 is mapped to the helix H and MHR region. The residues in bold are likely key to forming the epitope.

M10 peptide 151-176:

CLDIRQ GPKEP **FRDYV** **DRFYK** **TLRAEQ** (SEQ ID NO:3)

O10 peptide 152-177:

CLDIKQ GPKEP **PRDYV** **DRFYK** **TLRAEQ** (SEQ ID NO:4)

Monoclonal antibody 115B-303-620 bound to M12 and O12 peptides, which corresponds to the helix J-K region

of p24. The epitope appears to be linear based on the strong (high S/N) binding to free (un-conjugated) peptides of M12/O12. The residues in bold are likely key to forming the epitope, but secondary structure involving or requiring neighboring amino acids cannot be excluded.

M12 peptide:

CKTIL KALGP AATLE EMMTA (SEQ ID NO:5)

O12 peptide:

CKQIL KALGP GATLE EMMVA (SEQ ID NO:6)

Monoclonal antibody 120A-270-108 mapped to the helix H and MHR region of p24. Similar to monoclonal antibody 117-289-555. However, 120A-270-108 recognizes an epitope distinct from an epitope recognized by 117-289-555. The significant difference between 117-289-555 and 120A-270-108 is that 120A-270-108 only moderately bound to the KLH conjugated M10 peptide. No binding was detected when 120A-270-108 was reacted against free (un-conjugated) peptides. Thus, the optimal epitope of 120A-270-108 requires specific secondary or tertiary structures. Furthermore, 117-289-555 and 120A-270-108 belong to different compatibility groups because they bind simultaneously to core proteins without interference or competition from each other (see Table 7 below).

Table 7. Sandwich formation of mAbs with p24proteins
(both group M and group O)

MAbs in solid phase	Signal labeled mAbs in solution phase					
	103-350-474	117-289-555	115B-303-620	115B-151-423	108-394-470	120A-270-108
103-350-474	-	+	+	+	-	-

117-289-555	+	-	+	+	+	+
115B-303-620	+	+	-	+	+	+
115B-151-423	+	+	+	-	+	+
108-394-470	-	+	+	+	-	-
120A-270-108	-	+	+	+	-	-
120B-580-106 helix A	+	+	+	-	+/-	-

The + sign indicates compatibility of paired mAbs binding to p24 antigen simultaneously

5

The compatibility study also showed that, unlike 117-289 which can pair with either Mab 103-350 or 108-394 to form a sandwich, 120A-270 cannot form a sandwich with either Mab (Table 7). The compatibility data clearly demonstrated that the epitope recognized by 120A-270 is distinct and different from the epitope recognized by 117-289. Mab 120A-270 most likely recognizes a conformational epitope, formed in part, by amino acids 151-176.

15

Two Mabs 115B-151 and 108-394, fail to bind free synthetic peptides, and 115B-151 weakly bound to one KLH coupled peptide (Figure 2B). Failure to bind synthetic peptides indicated that these Mabs recognize conformational epitopes on core antigen. Conformational epitopes are formed by contiguous amino acids brought together by the tertiary or quaternary folding of p24 antigen. A tertiary or quaternary structure dependent

20

conformational epitope generally cannot be mimicked by small synthetic peptides.

A set of large overlapping p24 polypeptides were used to locate conformational epitopes recognized by the 115B-151 and 108-394. Overlapping p24 polypeptides were expressed in E. coli. from plasmids carrying unique portions of p24 nucleotide sequence (deletion clones). Two sets of six deletion clones were designed based on the structure of p24 (Figure 3). Specific binding of monoclonal antibodies to p24 polypeptides was determined using the Western blot method. Briefly, the expressed (recombinant) p24 polypeptides in extracts of E. coli were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes containing electrophoretically separated proteins were reacted with p24 Mabs (~ 5ug/ml concentration) and bound monoclonal antibodies were detected by enzyme labeled goat anti-mouse IgG. Figure 4 illustrates Western blot results of Mabs 115B-151 and 108-394.

Monoclonal antibody 115B-151 specifically bound to group M polypeptides F(1-172) and G(137-231) and group O polypeptides N(1-173) and O(138-232). The overlapping region (amino acids 137-172) between F and G or between N and O, may contribute to epitope recognized by 115B-151. This data is supported by weak but consistent binding of 115B-151 against the KLH conjugated synthetic peptides KM10 and KO10, which contain amino acids 137-172 (Figure 2B). Mab 115B-151 differed from 117-289 and 120A-270 because (a) 115B-151 apparently required the epitope to be in a specific secondary or tertiary conformation, (b) it was compatible with 117-289/120A-270 to form a sandwich with p24 (Table 7), and (c) monoclonal antibodies against helix A were incompatible and strongly competed with 115B-151, but were compatible with 117-289.

The strong competition of helix A directed monoclonal with 115B-151, in addition to the weak binding against KLH conjugated peptides, may indicate that the optimal epitope recognized by 115B-151 includes a portion of
 5 helix A in addition to the minimal linear epitope identified within helix H.

Monoclonal 108-394 mapped to a conformational epitope formed within the first 172/173 amino acids of M/O p24. The epitope is non-linear because HIV-1 M and O synthetic
 10 peptides, free or conjugated to carrier protein KLH, were unreactive with 108-394. In addition, 108-394 reacted only with the largest of the polypeptides (F 1-172/ N 1-173) derived from M/O p24. Although M polypeptides C (1-65), E (1-130), and I (60-150), and polypeptides L (1-
 15 65), M (1-131), and Q (60-151) contain large segments of the same sequence found in polypeptides F (1-172) and N (1-173), an epitope recognized by 108-394 apparently was not formed from the shorter polypeptides. These data are consistent with a conformational epitope formed by a
 20 major portion of p24 comprising at least the first 172/173 amino acids.

Based on the data from epitope mapping using synthetic peptides and p24 deletion clones and the data from the p24 sandwich compatibility study, an epitope map
 25 of the six mAbs on the three dimensional structure of p24 antigen is illustrated in Figure 5. The structure of the p24 molecule is represented by two domains, the N-terminal domain (amino acids 1-151) and the C-terminal domain (amino acids 151-231). The structure of the
 30 intact p24 molecule has not been determined so the exact structural relationship between the two domains is not fully characterized.

The six mAbs mapped to p24 antigen in the following manner (Figure 5):

Monoclonal antibody 103-350-474 binds a linear epitope located in the helix D region of HIV-1 M and O p24. The epitope is most broadly defined as comprising amino acids 63-89, and is most narrowly estimated as comprising amino acids 63-80 of group M and group O of the p24 antigen.

Monoclonal antibody 117-289-555 binds a linear epitope located in the MHR/helix H region of p24. The epitope is most broadly defined as comprising amino acids 151-172 (M)/ 152-173 (O), and is most narrowly defined as amino acids 162-172(M)/163-173(O) of p24 antigen.

Monoclonal antibody 115B-303-620 binds a linear epitope located in the helical J-K regions of p24 antigen. The epitope is defined as amino acids 198-217(M)/199-218(O) of the p24 antigen.

Monoclonal antibody 115B-151-423 binds a conformational epitope which is most likely near the junction part of helix A and MHR/helix H regions of p24.

Monoclonal antibody 108-394-470 binds a conformational epitope formed within the N-terminal domain (amino acids 1-151) of p24. The conformational epitope is estimated to be near the junction part of helix D and helix A.

Monoclonal antibody 120A-270-108 binds a conformational epitope which is estimated to be near the junction part of helix D, helix A and MHR/helix H regions of p24.

Example 9Preparation of monoclonal antibody coated microparticles

Carboxyl-modified latex (CML) microparticles at 1% solid (obtained from Bangs Laboratories, Fishers, IN) were activated by carbodiimide EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride from Sigma Chemicals, St. Louis, MO] at a molar ratio of EDC: carboxyl groups = 10:1 in 50 mM MES [2-(N-morpholino)ethane sulfonic acid] buffer pH 6.1 for 5 minutes at RT on a end-over-end rotator (Roto-Torque, Cole Parmer Instruments, Vernon Hills, IL) set at high on scale 5. Anti-core Mab was added to EDC pre-activated CML microparticles at a ratio of 200 ug antibody / per ml of 1% solid microparticles for 4 hours at room temperature on an end-over-end rotator. Free reactants were washed away using Abbott diafiltration system (Abbott Laboratories, Abbott Park, IL) with a crossflow syringe membrane (0.2 um pore size and 12 cm² surface area, obtained from Spectrum, Laguna Hills, CA). Mab-coated microparticles were overcoated with buffer containing 10 mM PBS and 5% BSA, 0.03% sodium azide for 1 hour at RT on a rotator. Mab-coated CML microparticles were heat-stressed in a 45 C oven incubator for 20 hours and then tested by an Abbott Prism Stand-alone instrument (Abbott Laboratories, Abbott Park, IL).

Example 10Preparation of Acridinium-Labeled Antibody Conjugates

Anti-HIV core Mabs in PBS, pH 7.4, reacted with acridinium-N-hydroxysuccinimide (ACR-NHS) active ester at the molar ratio of Mab:ACR-NHS = 1:15 for 10 minutes at RT on an end-over-end rotator (Cole Parmer Instruments, Vernon Hills, IL). ACR-labeled-Mab conjugate was separated from free reactants by a G-25 Sephardex column

(15 cm x 1.5 cm) which was pre-equilibrated in PBS pH 6.3 containing 1% CHAPS. The elution peak of ACR-labeled-Mab was monitored by following absorbance at 280nm using a spectrophotometer (Shimadzu UV-2101PC). The

- 5 concentration of ACR-labeled-mAb protein = $(OD_{280nm} - 0.247 \times OD_{370nm}) / 1.38$. Molar ratios of ACR / mAb = $[OD_{370nm} / (OD_{280nm} - 0.247 \times OD_{370nm})] \times 15$.

003327 000000

Example 11Prism Immunoassay Methods and Preparation

Anti-p24 mAb-microparticles (concentrate stock) were diluted in uParticle diluent (10 mM PBS, pH 6.5 containing 5% calf serum, 7.5% sucrose, 50 mM EDTA, 0.1% Tween 20, and 0.1% proclin). Anti-p24 mAb-ACR conjugates were diluted in conjugate diluent (10 mM PBS, pH 6.3 containing 40 mM EDTA, 5% calf serum, 0.5% Triton, and 0.1% proclin). Two wash buffers were used in the assay. Transfer wash buffer contained 25 mM MES pH 5.7, 150 mM NaCl, 4% Triton X-100, 1% Tween 20, 0.001% PEG, 0.1% proclin, and 0.001% antifoam. Conjugate wash buffer contained 10mM CAPS, pH 9.9, 150 mm NaCl, 5% Triton X-100, 0.1% proclin, and 0.001% antifoam. HIV-1 p24-M, rp24-O, HIV-2 rp26, and HIV-2 viral lysate were diluted in HIV-1/2 negative human plasma. Normal human plasma was also used as a negative control.

Assay procedure:

Briefly, all reagents were warmed up to room temperature before priming the instrument. Transfer wash buffer, conjugate wash buffer, anti-p24 mAb-ACR conjugate, and activator were connected to a proper reagent line. The reagents were primed two times and any air bubbles trapped in the reagent lines were tapped away. One hundred ul of samples and 50 ul of specimen diluent (25 mM PBS pH 6.5, 1% Triton X-100, 0.4% Tween 20, 20 mM EDTA, and 0.1% proclin) were added into channel A and B reaction wells of sample tray manually. The trays were loaded on the instrument and moved constantly through the channel during the assay steps. When the sample tray moved to the microparticle station, 50 ul of anti-p24 mAb-coated microparticles were added into each reaction well. The rest of the assay steps were

performed automatically by the instrument. The channel temperature of the instrument was maintained at 37 C. after 18 minutes incubation at 37 C, the sample tray was moved to the transfer wash station. The mixture of sample and uPrarticles were flushed onto a glass fiber matrix by transfer buffer and washed 2 times with transfer buffer. Fifty ul of anti-p24 mAb-ACR conjugate were added onto the glass fiber matrix. After 20 minutes incubation, the sample tray was moved to the conjugate wash station. Unbound conjugates were washed away by conjugate wash buffer. After the conjugate wash, the sample tray was moved to the activator station. Fifty ul of activator (mixture of hydrogen peroxide and sodium hydroxide) were applied to the matrix. The chemiluminescence light signal was read by a photomultiplier tube detector.

Example 12

Equivalent Detection of HIV core proteins (Antigens) Using Two Monoclonal Antibodies

Equivalent, quantitative core antigen sensitivity using compatible pairs of high affinity monoclonals was demonstrated in a two-step, chemiluminescent, "sandwich" immunoassay run on an Abbott Prism standalone instrument (Abbott Laboratories, Abbott Park, IL). By combining 120A-270-108 coated microparticles (0.066% solid) with 115B-151-423-ACR conjugate at 60 ng/ml, equivalent detection of HIV-1 group M, HIV-1 group O, and HIV-2 core proteins was achieved. The lowest limits of detection for HIV-1 group M p24 was estimated at 0.3 pg/ml (Figure 6), 0.3 pg/ml for HIV-1 group O rp24 (Figure 7), and 1.0 pg/ml for HIV-2 rp26 (Figure 8). Only a small (3.3 folds) differences in quantitative sensitivity was detected between HIV-1 and HIV-2. Equivalent sensitivity across

1. The first part of the document is a list of references. The references are listed in a vertical column, with the first reference at the top and the last reference at the bottom. The references are:

- 1. The first part of the document is a list of references. The references are listed in a vertical column, with the first reference at the top and the last reference at the bottom. The references are: